Docket No.: 11009/35975C
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Nancy Elise Gabriel and Christine E. Farrar

Application No.: 09/817,725 Confirmation No.: 1724

Filed: March 26, 2001 Art Unit: 1654

For: N-TERMINALLY CHEMICALLY MODIFIED PROTEIN COMPOSITIONS AND METHODS Examiner: B. D. Chism

DECLARATION UNDER 37 C.F.R. §1.131

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. I, Christine E. Farrar, declare that:

2. I am named as a co-inventor on United States Patent Application Serial No. 09/817,725. Nancy Elise Gabriel is also a co-inventor on the application.

3. When I was employed at Amgen, one of my responsibilities was working on a project that resulted in the production of hematopoietic proteins modified at the N-terminus with a polyethylene glycol moiety. I have been informed that such proteins are presently being claimed in the application.

4. The laboratory work I conducted that was associated with the project is described in my Amgen Notebook Nos. 5575, 5576 and 6951 attached as Exhibits A, B and C, respectively, to the "Declaration under 37 C.F.R. §1.131" of Nancy Elise Gabriel. Dates have been redacted from the exhibits. My notebook pages, specifically referenced below and dated prior to October 1993, demonstrate that we had produced and recognized the value of hematopoietic proteins modified at the N-terminus with a polyethylene glycol moiety.

5. Granulocyte colony-stimulating factor (G-CSF) was one of the hematopoietic proteins we were modifying with polyethylene glycol (PEG) prior to October 1993.

6. Recombinant human met-G-CSF (referred to as "rhG-CSF" or "rh-G-CSF" herein) was prepared according to methods in the Souza patent, U.S. Patent No. 4,810,643. The rhG-CSF had the amino acid sequence (encoded by the DNA sequence) shown below.

ATG ACT CCA TTA GGT CCT GCT TCT TCT CTG CCG CAA AGC TTT CTG
M T P L G P A S S L P Q S F L

CTG AAA TGT CTG GAA CAG GTT CGT AAA ATC CAG GGT GAC GGT GCT
L [K] C L E Q V R [K] I Q G D G A

GCA CTG CAA GAA AAA CTG TGC GCT ACT TAC AAA CTG TGC CAT CCG
A L Q E [K] L C A T Y [K] L C H P

GAA GAG CTG GTA CTG CTG GGT CAT TCT CTT GGG ATC CCG TGG GCT
E E L V L L G H S L G I P W A

CCG CTG TCT TCT TGT CCA TCT CAA GCT CTT CAG CTG GCT GGT TGT
P L S S C P S Q A L L Q A G C

CTG TCT CAA CTG CAT TCT GGT CTG TTC CTG TAT CAG GGT CTT CTG
L S Q L H S G L F L Y Q G L L

CAA GCT CTG GAA GGT ATC TCT CCG GAA CTG GGT CCG ACT CTG GAC
Q A L E G I S P E L G P T L D

ACT CTG CAG CTA GAT GTA GCT GAC TTT GCT ACT ACT ATT TGG CAA
T L Q L D V A D F A T T I W Q

CAG ATG GAA GAG CTC GGT ATG GCA CCA GCT CTG CAA CCG ACT CAA
Q M E E L G M A P A L Q P T Q

GGT GCT ATG CCG GCA TTC GCT TCT GCA TTC CAG CGT CGT GCA GGA
G A M P A F A S A F Q R R A G

GGT GTA CTG GTT GCT TCT CAT CTG CAA TCT TTC CTG GAA GTA TCT
G V L V A S H L Q S F L E V S

TAC CGT GTT CTG CGT CAT CTG GCT CAG CCG TAA TAG
Y R V L R H L A Q P * *

The rhG-CSF had one alpha amino reactive group (amino acid residue 1) and four epsilon amino reactive groups (amino acid residues boxed above) available for modification with a PEG moiety.

7. A 10 mg/ml solution of the above rh-G-CSF, in 100 mM Bicine pH 8.0, was added to solid SCM-MPEG (N-hydroxy succinimidyl esters of carboxymethyl methoxy polyethylene glycol) (Union Carbide) with an average molecular weight of 6000 Daltons to give a 1.5 molar excess of SCM-MPEG to rh-G-CSF. After one hour with gentle stirring, the mixture was diluted to 2 mg/ml with sterile water, and the pH was adjusted to 4.0 with dilute HCl. The reaction was carried out at room temperature. At this stage, the reaction mixture

consisted mainly of three forms of mono-pegylated rh-G-CSF, some di-pegylated rh-G-CSF, unmodified rh-G-CSF and reaction bi-product (N-hydroxy succinimide). See pages 82 and 83 of Notebook No. 5576.

8. The three forms of monopegylated rh-G-CSF were separated from each other using ion exchange chromatography. The reaction mixture was loaded (1 mg protein/ml resin) onto a Pharmacia S Sepharose FF column (Pharmacia XK50/30 reservoir, bed volume of 440 ml) equilibrated in buffer A (20 mM sodium acetate, pH 4.0). The column was washed with 3 column volumes of buffer A. The protein was eluted using a linear gradient from 0-23% buffer B (20 mM sodium acetate, pH 4.0, 1M NaCl) in 15 column volumes. The column was then washed with one column volume of 100% buffer B and reequilibrated with 3 column volumes of buffer A. The flow rate for the entire run was maintained at 8 ml/min. The eluent was monitored at 280 nm and fractions were collected. Fractions containing the individual monopegylated species were pooled according to Figure 1A of the patent application. See page 86 of Notebook No. 5576. These pools were concentrated with a 350 mL Amicon stirred cell using a YM10 76 mm membrane. See pages 83-89 of Notebook No. 5576.

9. Pooled fractions from the ion exchange chromatography were subjected to size exclusion chromatography to separate di-pegylated species from monopegylated species. Typically, 5-10 mg in 2-5 ml of solution were loaded onto a 120 ml Pharmacia Superdex 75 HR 16/60 column equilibrated with 20 mM sodium acetate pH 4.0. The column was run at 1.5 ml/min for 100 min. Two ml fractions were collected. The protein content of the eluent was monitored at 280 nm. Fractions from separated peaks were pooled. See pages 1-13 of Notebook No. 6951. Page 5 of the notebook reports enough material had been obtained so that the characteristics of the three forms of monopegylated rh-G-CSF could be analyzed.

10. As described in Example 1 of the patent application, five analyses were done to characterize the three forms: (1) SDS-Page, (2) Size exclusion chromatography HPLC ("SEC HPLC"), (3) peptide mapping analysis, (4) *in vitro* G-CSF bioassay, and (5) *in vivo* testing in hamster.

11. Peptide mapping and N-terminal sequence analyses (described at Application page 33, line 21 through page 36, line 3) revealed that one form, "Mono-PEG-1", was an N-terminally monopegylated conjugate. See pages 20 and 21 of Notebook No. 6951.

12. The N-terminally monopegylated rhG-CSF and the other two monopegylated samples were tested for activity in an *in vitro* mitogenic assay utilizing a G-CSF dependent

clone of murine 32D cells. Cells were maintained in Iscoves medium containing 5% FBS and 20 ng/ml rhG-CSF. Prior to sample addition, cells were prepared by rinsing twice with growth medium lacking rhG-CSF. An extended twelve point rhG-CSF standard curve was prepared, ranging from 48 to 0.5 ng/ml (equivalent to 4800 to 50 IU/ml). Four dilutions, estimated to fall within the linear portion of the standard curve, (1000 to 3000 IU/ml), were prepared for each sample and run in triplicate. Because of their apparent lower activity *in vitro*, the pegylated rhG-CSF samples were diluted approximately 4-10 times less. A volume of 40 µl of each dilution of sample or standard was added to appropriate wells of 96 well microtiter plate containing 10,000 cells/well. After forty-eight hours at 37°C and 5.5% CO_2, 0.5 µmCi of methyl-^{3}H-thymidine was added to each well. Eighteen hours later, the plates were then harvested and counted. A dose response curve (log rhG-CSF concentration vs. CPM-background) was generated and linear regression analysis of points which fall in the linear portion of the standard curve was performed. Concentrations of unknown test samples were determined using the resulting linear equation and correction for the dilution factor. As can be seen from Figure 4 of the patent application, of the three monopegylated species, N-terminally monopegylated G-CSF demonstrated the highest *in vitro* biological activity. The N-terminally monopegylated material had 68% of the activity of non-modified rhG-CSF. See page 17 of Notebook No. 6951.

13. *In vivo* testing confirmed the activity of the N-terminally monopegylated material. The *in vivo* testing was carried out by dosing male golden hamsters with a 0.1 mg/kg of sample, using a single subcutaneous injection. Four animals were subjected to terminal bleeds per group per time point. Serum samples were subject to a complete blood count on the same day that the samples were collected. The average white blood cell counts were calculated. As can be seen in Figures 5A and 5B of the patent application, the response from each material peaks after one day following a single subcutaneous injection of 0.1 mg/kg. Two of the monopegylated materials, (N-terminal and Lys-35) showed prolonged responses, while the response for the protein pegylated at lysine-41 showed no increase in *in vivo* activity over unmodified rhG-CSF (indeed it shows less, Application Figure 5B). See pages 18 and 19 and pages 74-77 of Notebook No. 6951.

14. We thus had made a hematopoietic protein modified at the N-terminus with a PEG moiety which had a prolonged *in vivo* biological activity as evidenced by the aforementioned notebook pages which are dated prior to October 1993.

15. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

1/11/06
Date

Christine E. Farrar
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